FORM PTO-1390 (Modified) (REV 11-98) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER	U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR)
PF95PCTSEQ/dln	09/936677

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/FR00/00623	15 MAR 2000 (12.03.00)	15 MAR 1999 (15.03.99)

TITLE OF INVENTION
IMMUNOSTIMULANT BACTERIAL MEMBRANE FRACTIONS IN CANCER TREATMENT

APPLICANT(S) FOR DO/EO/US
Christine LIBON, Nathalie CORVAIA, Alain BECK, Jean-Yves BONNEFOY

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☒ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ A copy of the International Search Report (PCT/ISA/210).
8. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☒ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.
9. ☒ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
10. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☒ Certificate of Mailing by Express Mail
20. ☒ Other items or information:

Sequence listing paper copy
Sequence listing disk copy
Statement by Attorney under 37 CFR §1.821(f)

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
09/936677	PCT/FR00/00623	PF95PCTSEQ/dln

531 Rec'd PCT/PTO 14 SEP 2001

21. The following fees are submitted:.	CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :		
☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1,000.00**		
☒ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but Internation Search Report prepared by the EPO or JPO **$860.00**		
☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$710.00**		
☐ International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$690.00**		
☐ International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	**$860.00**	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).	**$0.00**	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	33 - 20 =	13	x $18.00	**$234.00**	
Independent claims	1 - 3 =	0	x $80.00	**$0.00**	
Multiple Dependent Claims **(check if applicable)**.			☐	**$0.00**	
TOTAL OF ABOVE CALCULATIONS =				**$1,094.00**	
Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28) **(check if applicable)**. ☐				**$0.00**	
SUBTOTAL =				**$1,094.00**	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). +				**$0.00**	
TOTAL NATIONAL FEE =				**$1,094.00**	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).** ☐				**$0.00**	
TOTAL FEES ENCLOSED =				**$1,094.00**	
				Amount to be: refunded	$
				charged	$

☒ A check in the amount of **1,094** to cover the above fees is enclosed.

☐ Please charge my Deposit Account No. in the amount of to cover the above fees.
A duplicate copy of this sheet is enclosed.

☒ The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. **8-3220** A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

G. Patrick Sage
THE FIRM OF HUESCHEN AND SAGE
500 Columbia Plaza
350 East Michigan Ave.
Kalamazoo MI, 49007

G. Patrick Sage
SIGNATURE

G. Patrick Sage
NAME

37,710
REGISTRATION NUMBER

September 14, 2001
DATE

PF95PCTSEQ/dln

* * * * *

Applicant : Christine LIBON, et al.

Title : IMMUNOSTIMULANT BACTERIAL MEMBRANE FRACTIONS IN CANCER TREATMENT

* * * * *

Honorable Commissioner of Patents and Trademarks
Washington, D.C. 20231

STATEMENT UNDER 37 CFR 1.821(f)

The undersigned attorney does hereby state that, to the best of his knowledge and understanding, the accompanying Sequence Listing in computer readable form is the same as the accompanying Sequence Listing in paper copy form.

Respectfully submitted,

THE FIRM OF HUESCHEN AND SAGE

By: G. PATRICK SAGE
G. PATRICK SAGE, #37,710

Dated: September 14, 2001
Customer No: **25,666**
500 Columbia Plaza
350 East Michigan Avenue
Kalamazoo, MI 49007
616-382-0030

Enclosure: Sequence Listing in diskette form and paper form

PF95PCTSEQ/dln

* * * * *

Applicant : Christine Libon, et al.

Title : IMMUNOSTIMULANT BACTERIAL MEMBRANE FRACTIONS IN CANCER TREATMENT

* * * * *

Honorable Commissioner of Patents and Trademarks
Washington, D.C. 20231

PRELIMINARY AMENDMENT

Sir:

A soon as a Serial Number and Filing Date have been accorded the above-identified national phase application, kindly amend as follows:

IN THE CLAIMS: Kindly cancel all of the Claims, 1 through 28, and replace by Claims 29 through 61 as provided herewith.

R E M A R K S

The present application is a national phase filing of PCT/FR00/00623.

Applicants have cancelled all of the originally-filed Claims, 1 through 28. New Claims 29 through 61 have been added to better encompass the full scope and breadth of the invention notwithstanding Applicants' belief that the Claims would have been allowable as originally filed. Accordingly, Applicants assert that no Claims have been narrowed within the meaning of *Festo*.

Entry of the new Claims and early and favorable action on the merits of this application are respectfully solicited.

Respectfully submitted,

THE FIRM OF HUESCHEN AND SAGE

By: G. PATRICK SAGE

G. PATRICK SAGE

Dated: September 12, 2001
Customer No.: 25,666
500 Columbia Plaza
350 East Michigan Ave.
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Enclosure: Postal Card Receipt
Claims 29 through 61

PF95PCTSEQ/dln

We Claim:

- 29 -

The use of a membrane fraction of Gram-negative bacteria, comprising proteoglycans, for preparing a pharmaceutical composition which is immunostimulant and/or which is capable of inducing an antitumor immune response.

- 30 -

The use of Claim 29, wherein the membrane fraction comprises a membrane fraction of *Klebsiella pneumoniae*.

- 31 -

The use of Claim 29, wherein the membrane fraction comprises membrane fractions of at least two different strains of bacteria.

- 32 -

The use of Claim 29, wherein preparation of the membrane fraction comprises the following steps:

a) culturing the bacteria in a culture medium which allows their growth, followed by centrifugation of the culture;

b) where appropriate, deactivation of the lytic enzymes of the bacterial pellet obtained in step a), then centrifugation of the suspension obtained;

c) extraction and elimination of the non-membrane-bound proteins and of the nucleic acids of the pellet obtained in step a) or b) with at least one cycle of washing the pellet in an extraction solution;

d) digestion of the membrane pellet obtained in step c) in the presence of protease enzymes, followed by centrifugation;

e) at least one cycle of washing the pellet obtained in step d) in a physiological solution and/or in distilled water; and

f) ultrasonication of the pellet obtained in step e).

- 33 -

The use of Claim 29, wherein preparation of the membrane fraction comprises the following steps:

a) culturing of the bacteria in a culture medium which allows their growth followed, where appropriate, by centrifugation;

b) freezing of the culture medium or of the pellet obtained in step a), followed by thawing and drying of the cells;

c) elimination, using a DNase, of the nucleic acids from the dried cells obtained in step b), which have been resuspended;

d) grinding of the cells obtained in step c) and clarification of the suspension obtained;

e) precipitation, in acid medium, of the suspension obtained in step d) and elimination of the pellet;

f) neutralization of the supernatant obtained in step e) containing the membrane suspension, followed by dialysis and concentration of the membrane suspension; and

g) sterilization of the concentrated membrane suspension obtained in step f).

- 34 -

The use of Claim 29, wherein the pharmaceutical composition also comprises a vehicle agent for the membrane fraction in a form which makes it possible to improve its stability and/or its immunostimulant activity and/or its capacity to induce an antitumor immune response.

- 35 -

The use of Claim 34, wherein the agent is of the oil-in-water or water-in-oil emulsion type.

- 36 -

The use of Claim 34, wherein the agent is in the form of a particle of the liposome, microsphere or nanosphere type, or any type of structure which enables said membrane fraction to be encapsulated and presented in particulate form.

- 37 -

The use of Claim 29, wherein the pharmaceutical composition also comprises an agent for potentiating the immunostimulant activity and/or the antitumor immune response of said membrane fractions.

- 38 -

The use of Claim 37, wherein the agent for potentiating the immunostimulant activity and/or the antitumor immune response of said membrane fractions is a cytokine.

- 39 -

The use of Claim 37, wherein the agent for potentiating the immunostimulant activity and/or the antitumor immune response of said membrane fractions is a regulatory agent chosen from hormones.

- 40 -

The use of Claim 37, wherein the agent for potentiating the immunostimulant activity and/or the antitumor immune response of said membrane fractions is a regulatory agent chosen from growth factors.

- 41 -

The use of Claim 37, wherein the agent for potentiating the immunostimulant activity and/or the antitumor immune response of said membrane fractions is a cellular compound.

- 42 -

The use of Claim 41, wherein the cellular compound is a nucleic acid chosen from DNAs and RNAs.

- 43 -

The use of Claim 41, wherein the cellular compound is a compound of the ribosome family.

- 44 -

The use of Claim 41, wherein the cellular compound is a protein of the heat-shock protein family.

- 45 -

The use of Claim 29 for preparing pharmaceutical composition intended to be administered in combination with an anticancer treatment.

- 46 -

The use of Claim 45, wherein the anticancer treatment is chemotherapy and/or radiotherapy.

- 47 -

The use of Claim 45 for preparing a pharmaceutical composition intended to be administered simultaneously with, separately from, or at intervals with, the anticancer treatment.

- 48 -

The use of Claim 47, wherein the pharmaceutical composition is administered enterally or parenterally.

- 49 -

The use of Claim 45, wherein the combined anticancer treatment is a chemotherapeutic treatment comprising a protease inhibitor or a compound with anti-angiogenic activity.

- 50 -

The use of Claim 29 for preventing and/or treating cancers.

- 51 -

The use of Claim 50 for preventing and/or treating bladder cancers, prostate cancers, colon cancers, liver cancers and malignant melanomas.

- 52 -

A pharmaceutical composition comprising a membrane fraction of Gram-negative bacteria, comprising proteoglycans, which can be obtained using a method for preparing a membrane fraction of Claim 32.

- 53 -

A pharmaceutical composition comprising a membrane fraction of Gram-negative bacteria, comprising proteoglycans, which can be obtained using a method for preparing a membrane fraction of Claim 33.

- 54 -

The pharmaceutical composition of Claim 52, wherein the Gram-negative bacterium is *Klebsiella pneumoniae*.

- 55 -

The pharmaceutical composition of Claim 53, wherein the Gram-negative bacterium is *Klebsiella pneumoniae*.

- 56 -

The pharmaceutical composition of Claim 52 which is combined with an anticancer treatment by chemotherapy and/or by radiotherapy.

- 57 -

The pharmaceutical composition of Claim 53 which is combined with an anticancer treatment by chemotherapy and/or by radiotherapy.

- 58 -

The pharmaceutical composition of Claim 56 which contains an anticancer compound as a combination product for use which is simultaneous, separate, or at intervals.

- 59 -

The pharmaceutical composition of Claim 57 which contains an anticancer compound as a combination product for use which is simultaneous, separate, or at intervals.

- 60 -

The pharmaceutical composition of Claim 58, wherein the anticancer compound is chosen from protease inhibitors or from compounds with anti-angiogenic activity.

- 61 -

The pharmaceutical composition of Claim 59, wherein the anticancer compound is chosen from protease inhibitors or from compounds with anti-angiogenic activity.

IMMUNOSTIMULANT BACTERIAL MEMBRANE FRACTIONS IN THE TREATMENT OF CANCERS

The present invention relates to the use of a membrane fraction of Gram-negative bacteria, in particular of Klebsiella *pneumoniae*, for preparing a pharmaceutical composition which is immunostimulant and/or capable of inducing an antitumor immune response and which is intended, in particular, for treating and preventing cancers. The invention also comprises methods for preparing said membrane fractions and also pharmaceutical compositions containing them, in particular combined with anticancer compounds.

The transformation of a normal cell into a malignant cell is the result of many different events which may occur spontaneously, such as mutations or gene rearrangements, or be induced by chemical, physical or viral agents.

Tumors are infiltrated by immunocompetent cells, in particular lymphocytes, dendritic cells and macrophages.

Tumor-associated macrophages (TAMs) originate from the blood circulation and are recruited to the tumor site by cytokines. TAMs bind to the tumor cells via glycoproteins, sugars and phospholipids and proliferate at the tumor site (J. Natl. Cancer Inst., 1998, 90:1583). There, they secrete many cytokines which contribute to their antitumor activity. Among the most important are TNF-α and IL-12.

The antitumor activity of TNF-α has been demonstrated in experimental models in mice (Beyaert R. and Fiers W., Cytokines, chapter 24, 335-360 Academic Press. 1998) and has been tested in humans for treating bladder cancers: alone, it has moderate activity

(Steinberg et al., Ann. Oncol., 1992, 3,741-745; Eur. Urol. 1992, 22:112).

The production of IL-12 by activated macrophages serves to modulate the immune response by promoting the formation of Th1-type CD4+ T lymphocytes which produce IL-2 and IFN-γ. The inhibitory activity of IL-12 on angiogenesis and tumor regression is well known and appears to be linked to the induction of IFN-γ, which stimulates the production of IP-10 (interferon-inducible protein-10) and of MIG (monokine induced by IFN-γ) (J. Natl. Cancer Inst., 1998, 90:1583).

BCG (Bacille Calmette Guérin) therapy is used to prevent the recurrence of certain types of bladder cancer. The mechanism of action currently proposed is based on the production of cytokines: early release of inflammatory cytokines (TNF-α, IL-1, IL-6, IL-8) and, secondly, production of IL-2 and of IFN-γ (Th1 response), then later of IL-4, of IL-5 and of IL-10 (Th2 response). Finally, there occurs a phase of cell activation with amplification of cytotoxic populations (Patard et al., Progrès en Urologie, 1998, 8,415-421).

However, BCG therapy does not only have advantages, since the effectiveness sometimes observed is at the expense of a morbidity which is also greater. In addition, there are contraindications for BCG therapy: active tuberculosis (but not prior tuberculosis), immunosuppression (HIV, transplantation, etc.), prior systemic reaction to BCG (hepatitis, pneumonia, BCGitis), steroid treatments. Furthermore, resistances or recurrences exist after BCG therapy.

The membrane fraction of K. pneumoniae I145 goes into the composition of a pharmaceutical preparation which prevents the occurrence and recurrence of respiratory infections of bacterial origin and which has been used in humans for 20 years. For this reason, there has been

enough time to assess the nontoxicity of the product. The set of data cited above shows that there exists, today, a need to have novel immunostimulants free of toxic activity. Such immunostimulants would be of great value for treating certain types of cancer.

Surprisingly, the authors of the present invention have demonstrated that membrane fractions of a Gram-negative bacterium, especially Klebsiella *pneumoniae* (named FMKp), in particular membrane fractions obtained using the methods as described hereinafter in the examples, have the desired immunostimulant properties.

The inventors have shown, surprisingly, that the FMKp or one of its major constituents, the OmpA outer membrane protein named P40 (as described in patent applications WO 95/27787 and WO 96/14415) is capable not only of stimulating the proliferation of human blood mononucleated cells, thus demonstrating its immunostimulant activity, but also of inducing, in particular by monocytes, the production of TNF-α and of IL-12, which are cytokines involved in the antitumor immune response.

Thus, the subject of the present invention is the use of a membrane fraction of Gram-negative bacteria, in particular of Klebsiella *pneumoniae*, as a compound which is immunostimulant and/or capable of inducing an antitumor immune response, or for preparing a pharmaceutical composition which is immunostimulant and/or capable of inducing an antitumor immune response, this being whatever the mode of administration in vivo chosen (enteral or parenteral route).

In the present invention, the term "immunostimulant compound" or "immunostimulant pharmaceutical composition" is intended to denote a compound, or a

pharmaceutical composition, capable of increasing a nonspecific immune response.

In the present invention, the expression "compound capable of inducing an antitumor immune response" or "pharmaceutical composition capable of inducing an antitumor immune response" is intended to denote a compound, or a pharmaceutical composition, capable, in particular, of increasing the effectiveness of an anticancer compound or increasing the effectiveness of an anticancer treatment, such as for example treatment by radiotherapy.

The invention also relates to the use as claimed in the invention, characterized in that the membrane fraction comprises at least membrane fractions of two different strains of bacteria.

In the present invention, the expression "membrane fraction of a bacterium" is intended to denote any purified or partially purified membrane fraction or extract which is obtained from a culture of said bacterium and for which the method of preparation comprises at least one step for lysing the bacteria obtained after culturing and one step for separating the fraction containing the membranes of said bacteria from the total lysate obtained after the lysis step, in particular by centrifugation or filtration.

In the present invention, the expression "membrane fraction of a bacterium when said bacterium is Klebsiella *pneumoniae*" is also intended to denote the P40 protein, which is the active fraction of the membrane fraction of Klebsiella *pneumoniae*, of amino acid sequence SEQ ID No. 2, or a fragment thereof.

According to the invention, the membrane fractions may be prepared according to the methods known to those skilled in the art, such as for example the method

described by Haeuw J.F. et al. (Eur. J. Biochem, 255, 446-454, 1998).

According to one particular embodiment, the invention relates to a use as claimed in the invention, characterized in that the membrane fraction is prepared using a method comprising the following steps:

a) culturing of said bacteria in a culture medium which allows their growth, followed by centrifugation of said culture;
b) where appropriate, deactivation of the lytic enzymes of the bacterial pellet obtained in step a), then centrifugation of the suspension obtained;
c) extraction and elimination of the non-membrane-bound proteins and of the nucleic acids of the pellet obtained in step a) or b) with at least one cycle of washing the pellet in an extraction solution;
d) digestion of the membrane pellet obtained in step c) in the presence of proteolytic enzymes, followed by centrifugation;
e) at least one cycle of washing the pellet obtained in step d) in a physiological solution and/or in distilled water; and
f) ultrasonication of the pellet obtained in step e).

Step b) for deactivating the lytic enzymes of the bacterial pellet obtained in step a) may be carried out using any known method for deactivating enzymes, such as, in particular, by heating the resuspended bacterial pellet to a temperature preferably close to 100°C, or by adding an inhibitor of the activity of these enzymes.

Step c) for extracting and eliminating the non-membrane-bound proteins and the nucleic acids of the pellet obtained in step a) or b) may be carried out, for example, with at least one cycle of washing the pellet in an extraction solution corresponding to the

addition of a hypertonic solution (extraction solution), preferably a saline solution with a molarity close to 1 M, followed, after a period of contact sufficient for the desired effect, by centrifugation of the suspension obtained and elimination of the supernatant obtained after said centrifugation, this washing cycle possibly being reproduced several times.

Step d) for digesting the membrane pellet obtained in step c) may be carried out in the presence of a solution of proteolytic enzymes, such as for example trypsin, chymotrypsin or any known enzyme with proteolytic activity, the conditions of the reaction, pH of the solution, and temperature and duration of the reaction preferably being adjusted to the optimal conditions for the activity of the enzyme(s) chosen, followed by a centrifugation, this digestion cycle possibly being reproduced several times with the same enzyme or the same combination of enzymes, or with a different enzyme for each digestion cycle carried out.

Step e) for washing the pellet obtained in step d) is carried out by taking the pellet up in a physiological solution or in distilled water, followed, after a sufficient period of contact, by a centrifugation, this washing cycle possibly being reproduced several times.

Finally, the objective of step f) for ultrasonicating the pellet is, in particular, to disintegrate and homogenize the membrane fraction obtained at the end of step e). The ultrasonication conditions (duration and power) will be determined by those skilled in the art depending, for example, on the amount of membrane fraction to be treated.

According to another particular embodiment, the invention relates to a use as claimed in the invention, characterized in that the membrane fraction is prepared using a method comprising the following steps:

a) culturing of said bacteria in a culture medium which allows their growth, followed, where appropriate, by centrifugation;

b) freezing of the culture medium or of the pellet obtained in step a), followed by thawing and drying of the cells;

c) elimination, using a DNase, of the nucleic acids from the dried cells obtained in step b), which have been resuspended;

d) grinding of the cells obtained in step c) and clarification of the suspension obtained;

e) precipitation, in acid medium, of the suspension obtained in step d) and elimination of the pellet;

f) neutralization of the supernatant obtained in step e) containing the membrane suspension, followed by dialysis and concentration of the membrane suspension; and

g) sterilization of the concentrated membrane suspension obtained in step f).

The conditions for freezing in step b) of the method below will, of course, be determined by those skilled in the art depending on the initial amount of pellet to be treated, preferably carried out at 4°C for at least 48 hours for the equivalent of 1 kg of dried cells.

In step c), the nucleic acids are eliminated, for example, by adding a DNase at a final concentration of 5 mg/ml of a suspension of cells at a concentration equivalent to 5% of dried cells.

The grinding of the cells obtained in step c) may be carried out using any system or apparatus known to those skilled in the art for grinding cells, such as presses or preferably such as Manton Gaulinet loop grinding for 30 minutes.

The clarification of the suspension obtained after grinding may be carried out using any system or apparatus known to those skilled in the art for clarifying ground bacterial cell material, such as the Sharpless system.

Step e) for precipitating, in acid medium, the suspension obtained in step d) may be carried out, for example, with acetic acid. The precipitation is followed by elimination of the pellet using, for example, a system of the Sharpless type and by recovery of the supernatant.

Step f) consists of a step in which the supernatant, obtained after precipitation in acid medium, is neutralized, diluted, dialyzed and then concentrated.

Finally, the last step consists of a step for sterilizing the membrane fraction concentrate obtained in the preceding step, for instance by heating at 121°C for approximately 35 minutes, for example.

The invention relates particularly to the use as claimed in the invention, characterized in that the membrane fraction is the Klebsiella *pneumoniae* P40 protein of sequence SEQ ID No. 2, a fragment thereof or a homologous protein, the sequence of which exhibits a percentage identity of at least 80%, preferably 90%, 95% and 99%, with the sequence SEQ ID No. 2, said fragments or said homologous protein being capable of inducing immunostimulant and/or antitumor activity.

For the purposes of the present invention, the term "percentage identity", "degree of identity" or "level of identity" between two nucleic acid or amino acid sequences is intended to denote a percentage of nucleotides or of amino acid residues which are identical between the two sequences to be compared, obtained after the best alignment, this percentage

being purely statistical and the differences between the two sequences being distributed randomly and over their entire length. Sequence comparisons between two nucleic acid or amino acid sequences are conventionally carried out by comparing these sequences after having optimally aligned them, said comparison being carried out by segment or by "window comparison" in order to identify and compare local regions of sequence similarity. The optimal alignment of the sequences for comparison may be produced, besides manually, by means of the local homology algorithm of Smith and Waterman (1981) [Ad. App. Math. 2:482], by means of local homology algorithm of Neddleman and Wunsch (1970) [J. Mol. Biol. 48:443], by means of the similarity search method of Pearson and Lipman (1988) [Proc. Natl. Acad. Sci. USA 85:2444], by means of computer software using these algorithms (GAP, BESTFIT, FASTA and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI, or with the BLAST N or BLAST P comparison software).

The percentage identity between two nucleic acid or amino acid sequences is determined by comparing these two sequences which have been optimally aligned by window of comparison in which the region of the nucleic acid or amino acid sequence to be compared may comprise additions or deletions with respect to the reference sequence for optimal alignment between these two sequences. The percentage identity is calculated by determining the number of identical positions for which the nucleotide or amino acid residue is identical between the two sequences, dividing this number of identical positions by the total number of positions in the window of comparison and multiplying the result obtained by 100, so as to obtain the percentage identity between these two sequences.

For example, use may be made of the BLAST program, "BLAST 2 sequences", which is available on the site

http://www.ncbi.nlm.nih.gov/gorf/bls.html, the parameters used being those given by default (in particular, for the "open gap penalty" parameter :5 and the "extension gap penalty" parameter :2; the matrix chosen being, for example, the "BLOSUM 62" matrix proposed by the program), the percentage identity between the two sequences to be compared being calculated directly by the program.

The expression "fragment of P40 protein" is intended to denote, in particular, any fragment of amino acid sequence included in the amino acid sequence of the P40 protein, which is capable of increasing a nonspecific immune response and/or capable of inducing an antitumor immune response, and which comprises at least 5 amino acids, preferably at least 10 amino acids, or more preferably at least 15 amino acids.

Of course, said P40 protein, or fragments thereof, may be obtained by chemical synthesis or in the form of recombinant peptides.

The methods for preparing recombinant peptides are, today, well known to those skilled in the art and will not be developed in the present description. Among the cells which may be used for producing these recombinant peptides, mention should, of course, be made of bacterial cells (Olins P.O. and Lee S.C., 1993, Recent advances in heterologous gene expression in E. coli. Curr. Op. Biotechnology 4:520-525), but also yeast cells (Buckholz R.G., 1993, Yeast Systems for the Expression of Heterologous Gene Products. Curr. Op. Biotechnology 4:538-542), as well as animal cells, in particular mammalian cell cultures (Edwards C.P. and Aruffo A., 1993, Current applications of COS cell based transient expression systems. Curr. Op. Biotechnology 4, 558-563), but also insect cells in which methods implementing, for example, baculoviruses may be used (Luckow V.A., 1993, Baculovirus systems for the

expression of human gene products. Curr. Op. Biotechnology 4, 564-572).

A subject of the invention is also the use as claimed in the invention, characterized in that the pharmaceutical composition also comprises an agent for vehiculing said membrane fraction in a form which makes it possible to improve its stability and/or its immunostimulant activity and/or its capacity to induce an antitumor immune response, such as in the form of an emulsion of the oil-in-water or water-in-oil type, or in the form of a particle of the liposome, microsphere or nanosphere type, or any type of structure which enables said membrane fraction to be encapsulated and presented in particulate form.

Also included in the present invention is the use as claimed in the invention, characterized in that the pharmaceutical composition also comprises an agent for potentiating the immunostimulant activity and/or the antitumor immune response of said membrane fractions.

Among said agents for potentiating the immunostimulant activity and/or the antitumor immune response of said membrane fractions, cytokines and cellular compounds are preferred.

Among cytokines, mention may be made, without being limited thereto, of: IL-2, IL-12, IL-18, IFN-γ and IFN-α.

Among cellular compounds, nucleic acids, compounds of the ribosome family or proteins of the heat-shock protein family are in particular preferred.

Also included in the present invention is the use as claimed in the invention, characterized in that the pharmaceutical composition also comprises a potentiating agent which makes it possible to regulate

the immunostimulant activity and/or the antitumor immune response of said membrane fractions.

Among said potentiating agents which make it possible to regulate the immunostimulant activity and/or the antitumor immune response of said membrane fractions, hormones and growth factors are preferred.

Among hormones, mention may be made, but without being limited thereto, of β-hCG.

Among growth factors, mention may be made, but without being limited thereto, of: EGF, IGF-1, IGF-2, GM-CSF and G-CSF.

The subject of the invention is also the use as claimed in the invention, for preparing a pharmaceutical composition intended to be administered in combination with an anticancer treatment, in particular an anticancer treatment by chemotherapy (mono- or polychemotherapy) and/or radiotherapy.

According to the invention, the preparation of the pharmaceutical composition is intended to be administered via the enteral or parenteral route, and simultaneously with, separately from or spread out over time with the anticancer treatment.

The invention also comprises the use as claimed in the invention, for preparing a pharmaceutical composition comprising a compound with anticancer activity combined with said membrane fraction.

Many compounds with anticancer activity may thus be combined with said membrane fraction which is immunostimulant and/or capable of inducing an antitumor immune response.

Among these compounds, mention may in particular be made, but without being limited thereto, of protease inhibitors or compounds with anti-angiogenic activity, such as for example:

- protease inhibitors such as TIMPs;

or the following compounds with anti-angiogenic activity: angiostatin, endostatin, MCP-1, IP-10 and PF-4, and also anti-VEGF, anti-angiogenin, anti-aFGF and anti-bFGF antibodies, antisense sequences or peptides.

Thus, the invention relates to the use as claimed in the invention, characterized in that said combined anticancer treatment is a chemotherapeutic treatment comprising a protease inhibitor or a compound with anti-angiogenic activity.

The subject of the invention is also the use as claimed in the invention, for preparing a pharmaceutical composition intended to prevent or treat cancers, in particular bladder cancers, prostate cancers, colon cancers, liver cancers or malignant melanomas.

In another aspect, the invention relates to a method for preparing a membrane fraction of Gram-negative bacteria, in particular Klebsiella *pneumoniae*, characterized in that it comprises the following steps:

a) culturing of said bacteria in a culture medium which allows their growth, followed by centrifugation of said culture;
b) where appropriate, deactivation of the lytic enzymes of the bacterial pellet obtained in step a), then centrifugation of the suspension obtained;
c) extraction and elimination of the non-membrane-bound proteins and of the nucleic acids of the pellet obtained in step a) or b) with at least one cycle of washing the pellet in an extraction solution;

d) digestion of the membrane pellet obtained in step c) in the presence of protease enzymes, followed by centrifugation;
e) at least one cycle of washing the pellet obtained in step d) in a physiological solution and/or in distilled water; and
f) ultrasonication of the pellet obtained in step e).

The invention also comprises the method for preparing a membrane fraction of Gram-negative bacteria, in particular Klebsiella *pneumoniae*, characterized in that it comprises the following steps:

a) culturing of said bacteria in a culture medium which allows their growth, followed, where appropriate, by centrifugation;
b) freezing of the culture medium or of the pellet obtained in step a), followed by thawing and drying of the cells;
c) elimination, using a DNase, of the nucleic acids from the dried cells obtained in step b), which have been resuspended;
d) grinding of the cells obtained in step c) and clarification of the suspension obtained;
e) precipitation, in acid medium, of the suspension obtained in step d) and elimination of the pellet;
f) neutralization of the supernatant obtained in step e) containing the membrane suspension, followed by dialysis and concentration of the membrane suspension; and
g) sterilization of the concentrated membrane suspension obtained in step f).

The membrane fractions which can be obtained using said methods of course form part of the invention.

The titer of proteoglycan of the membrane fractions which can be obtained using said methods, which proteoglycan is the active principle of the FMKp, which

titer is represented by the sum of the galactose and protein contents, is preferably:
- for the galactose : between 1.2 g/l and 3.4 g/l;
- for the proteins : between 7.5 g/l and 14.9 g/l.

More preferably, this titer will be:
- for the galactose : between 1.8 g/l and 2.6 g/l;
- for the proteins : between 9.3 g/l and 11.7 g/l.

The invention also relates to the pharmaceutical compositions comprising a membrane fraction which can be obtained using the methods as claimed in the invention.

Also included in the present invention are the pharmaceutical compositions comprising a membrane fraction of a Gram-negative bacterium, in particular of Klebsiella *pneumoniae*, characterized in that it is combined with an anticancer treatment by chemotherapy and/or by radiotherapy.

The term "membrane fraction" is herein intended to denote any membrane fraction of the Gram-negative bacterium as defined above, including that which can be obtained using the methods as claimed in the invention and the P40 protein or a fragment thereof.

Preferably, the invention relates to a pharmaceutical composition as claimed in the invention, characterized in that it contains an anticancer compound as a combination product for use which is simultaneous, separate or spread out over time, in particular an anticancer compound chosen from protease inhibitors or from compounds having anti-angiogenic activity.

Preferably, said pharmaceutical compositions as claimed in the invention may also comprise agents such as vehicles, agents capable of potentiating and/or of regulating the immunostimulant activity and/or the

antitumor immune response of said membrane fractions as defined above.

The legends to the figures and examples which follow are intended to illustrate the invention without in any way limiting the scope thereof.

Legends to the figures:

Figure 1 : Proliferation of PBMC in the presence of FMKp - Dose-response study

The mononucleated cells (PBMC) are obtained by separation with the aid of a solution of Ficoll-sodium metrizoate, using total blood. The PBMC are then seeded in a proportion of 10 000 cells/well in the presence of stimulating agents, in a total volume of 200 µl. After incubation for 72 h, the proliferation is objectified by adding tritiated thymidine. The results are expressed as stimulation index = [cpm PBMC + stimulus]/ [cpm PBMC without stimulus (= RPMI medium + 10% SVF)].

Figure 2 : Proliferation of PBMC in the presence of FMKp - Reproducibility of the effect on several donors (FMKp at 250 µg/ml).

Figure 3 : Production of TNF-α by blood monocytes

The monocytes are cultured in RPMI 1640 medium + 10% SVF and in the presence of various concentrations of product. The cells are incubated in an incubator at 37°C in an atmosphere containing 5% of CO_2. Culture conditions : 200 000 cells/well, incubation for 18 h. After incubation, the culture plates are centrifuged and the supernatants are aliquoted and stored at -80°C until they are assayed. The concentrations of cytokines present in the culture supernatants are determined by ELISA (Enzyme-Linked ImmunoSorbent Assay) : Predicta kit from Genzyme (detection threshold at 3 pg/ml).

Figure 4 : Production of IL-12 p70 (biologically active) by blood monocytes.

The monocytes are cultured in RPMI 1640 medium + 10% SVF and in the presence of various concentrations of product. The cells are incubated in an incubator at 37°C in an atmosphere containing 5% of CO_2. Culture conditions : 500 000 cells/well, incubation for 24 h. After incubation, the culture plates are centrifuged and the supernatants are aliquoted and stored at -80°C until they are assayed. The concentrations of cytokines present in the culture supernatants are determined by ELISA : Endogen antibody pair (detection threshold at 15 pg/ml).

Example 1 : Production of the membrane fraction of K.*pneumoniae* (FMKp)

Method No. 1

The extraction of the K. pneumoniae I145 membranes from the centrifugation pellet from the step is preferably preceded by a step for destroying the lytic enzymes of the cellular components contained in the pellet, for example by heating the pellet to 100°C, optionally after redissolving it.

The actual extraction of the membranes from the centrifugation pellet is preferably carried out by treating the cellular components of the pellet, after optional destruction of the lytic enzymes, with a saline solution, for example 1 M sodium chloride, one or more times, then centrifuging the suspension obtained, preferably at 20 000 g; the supernatant from this centrifugation, which is eliminated, contains the nonmembrane impurities such as proteins and nucleic acids, while the pellet contains the membranes.

After separation of the saline solution containing the impurities, the membranes are digested in the presence of proteolytic enzymes, preferably trypsin and chymotrypsin, in solution at pH 8, at 37°C for 4 hours.

After digestion, the solution is homogenized by ultrasonication. The product thus obtained constitutes the membrane fraction named FMKp.

The supernatant obtained is centrifuged again under the same conditions, preferably at 140 000 g.

Preparation of the membrane-bound glycopeptides

This fraction is prepared from the pellet obtained by centrifugation at 40 000 g for 20 minutes. Said pellet is resuspended in physiological saline and then this suspension is brought to 100°C for 10 minutes in a waterbath of boiling water so as to deactivate the lytic enzymes. After cooling, the suspension is centrifuged for 30 min at 20 000 g. The pellet obtained is extracted twice with 1M NaCl in order to eliminate the proteins and the nucleic acids. The membranes are recovered by centrifugation for 30 minutes at 20 000 g.

They are then subjected to digestion by trypsin at pH 8 and at 37°C for 4 hours, then by chymotrypsin under the same conditions.

The membranes are then recovered by centrifugation at 2 000 g for 30 minutes, washed with physiological saline and then distilled water and subjected to 15-minute disintegration by ultrasound.

Method No. 2

After thawing at +4°C for a minimum of 48 h, 1 kg of dry K. pneumoniae cells is resuspended at 5% dry cells. DNase is added at 5 mg/l. Next, Manton Gaulin loop grinding is carried out for 30 min, followed by a clarification of a Sharples at 50 l/h, and then

precipitation with acetic acid at pH = 4.2 + 0.1 for 30 min. The pellet is eliminated (Sharples at 25 l/h) and the supernatant is neutralized and diluted to twice the initial volume with osmosed water. Dialysis at constant volume is then performed on PUF 100 up to 800 Ωcm, followed by concentration of the membrane suspension (MS) thus obtained, to 11 l/kg of dry cells. The MS is then autoclaved at +121°C for 35 min and can be stored at +4°C for 6 weeks.

Characteristics of the FMKp

By definition, the titer of proteoglycan, which is the active principle of the FMKp, is equal to the sum of the galactose and protein contents.

- galactose : on average 2.2 g/l
- proteins : on average 10.5 g/l

Example 2 : Proliferation of PBMC from human blood

The results obtained show that, surprisingly, the FMKp triggers PBMC proliferation. This effect is dose-dependent and maximal for 2.5 mg/ml of FMKp (Figure 1). Moreover, this effect is reproducible (Figure 2).

Example 3 : Production of cytokines by monocytes purified from human blood

Human monocytes are obtained from the mononucleated cells (lymphocytes, monocytes, NK cells, etc.) isolated beforehand from total human blood. The production of monocytes is based on the expression, in large amount, of the CD14 surface antigen on the cells. The separation is a positive selection. The effectiveness of the magnetic separation of the monocytes is then evaluated by flow cytometry, labeling with a fluorescein isothiocyanate (FITC) - coupled CD13 antibody: the cell suspension then contains 94 to 97% of monocytes.

The results from in vitro studies demonstrate that, interestingly, the FMKp is an immunostimulant which

induces the proliferation of PBMC from human blood with a direct effect on the monocytes : production of TNF-α (Figure 3) and of IL-12 p70 (Figure 4). It is noteworthy that the recombinant P40 protein (rP40), the OmpA of K. pneumoniae, is also capable of stimulating the production of TNF-α (Figure 3) and of IL-12 p70 (Figure 4) by human monocytes.

CLAIMS

1/ The use of a membrane fraction of Gram-negative bacteria, comprising proteoglycans, for preparing a pharmaceutical composition which is immunostimulant and/or which is capable of inducing an antitumor immune response.

2/ The use as claimed in claim 1, characterized in that the membrane fraction comprises a membrane fraction of Klebsiella *pneumoniae*.

3/ The use as claimed in claim 1 or 2, characterized in that the membrane fraction comprises at least membrane fractions of two different strains of bacteria.

4/ The use as claimed in one of claims 1 to 3, characterized in that the membrane fraction is prepared using a method comprising the following steps:

a) culturing said bacteria in a culture medium which allows their growth, followed by centrifugation of said culture;

b) where appropriate, deactivation of the lytic enzymes of the bacterial pellet obtained in step a), then centrifugation of the suspension obtained;

c) extraction and elimination of the non-membrane-bound proteins and of the nucleic acids of the pellet obtained in step a) or b) with at least one cycle of washing the pellet in an extraction solution;

d) digestion of the membrane pellet obtained in step c) in the presence of protease enzymes, followed by centrifugation;

e) at least one cycle of washing the pellet obtained in step d) in a physiological solution and/or in distilled water; and

f) ultrasonication of the pellet obtained in step e).

5/ The use as claimed in one of claims 1 to 3, characterized in that the membrane fraction is prepared using a method comprising the following steps:

a) culturing of said bacteria in a culture medium which allows their growth, followed, where appropriate, by centrifugation;
b) freezing of the culture medium or of the pellet obtained in step a), followed by thawing and drying of the cells;
c) elimination, using a DNase, of the nucleic acids from the dried cells obtained in step b), which have been resuspended;
d) grinding of the cells obtained in step c) and clarification of the suspension obtained;
e) precipitation, in acid medium, of the suspension obtained in step d) and elimination of the pellet;
f) neutralization of the supernatant obtained in step e) containing the membrane suspension, followed by dialysis and concentration of the membrane suspension; and
g) sterilization of the concentrated membrane suspension obtained in step f).

6/ The use as claimed in one of claims 1 to 5, characterized in that the pharmaceutical composition also comprises an agent for vehiculing said membrane fraction in a form which makes it possible to improve its stability and/or its

immunostimulant activity and/or its capacity to induce an antitumor immune response.

7/ The use as claimed in claim 6, characterized in that said agent is of the oil-in-water or water-in-oil emulsion type.

8/ The use as claimed in claim 6, characterized in that said agent is in the form of a particle of the liposome, microsphere or nanosphere type, or any type of structure which enables said membrane fraction to be encapsulated and presented in particulate form.

9/ The use as claimed in one of claims 1 to 8, characterized in that the pharmaceutical composition also comprises an agent for potentiating the immunostimulant activity and/or the antitumor immune response of said membrane fractions.

10/ The use as claimed in claim 9, characterized in that the agent for potentiating the immunostimulant activity and/or the antitumor immune response of said membrane fractions is a cytokine.

11/ The use as claimed in claim 9, characterized in that the agent for potentiating the immunostimulant activity and/or the antitumor immune response of said membrane fractions is a regulatory agent chosen from hormones.

12/ The use as claimed in claim 9, characterized in that the agent for potentiating the immunostimulant activity and/or the antitumor immune response of said membrane fractions is a regulatory agent chosen from growth factors.

13/ The use as claimed in claim 9, characterized in that the agent for potentiating the immunostimulant activity and/or the antitumor immune response of said membrane fractions is a cellular compound.

14/ The use as claimed in claim 13, characterized in that said cellular compound is a nucleic acid chosen from DNAs and RNAs.

15/ The use as claimed in claim 13, characterized in that said cellular compound is a compound of the ribosome family.

16/ The use as claimed in claim 13, characterized in that said cellular compound is a protein of the heat-shock protein family.

17/ The use as claimed in one of claims 1 to 16, for preparing a pharmaceutical composition intended to be administered in combination with an anticancer treatment.

18/ The use as claimed in claim 17, characterized in that the anticancer treatment is chemotherapy and/or radiotherapy.

19/ The use as claimed in either of claims 17 and 18, for preparing a pharmaceutical composition intended to be administered simultaneously with, separately from or spread out over time with the anticancer treatment.

20/ The use as claimed in claim 19, characterized in that the pharmaceutical composition is administered via the enteral or parenteral route.

21/ The use as claimed in one of claims 17 to 20, characterized in that said combined anticancer treatment is a chemotherapeutic treatment comprising a protease inhibitor or a compound with anti-angiogenic activity.

22/ The use as claimed in one of claims 1 to 21, for preventing and/or treating cancers.

23/ The use as claimed in claim 22, for preventing and/or treating bladder cancers, prostate cancers, colon cancers, liver cancers and malignant melanomas.

24/ A pharmaceutical composition comprising a membrane fraction of Gram-negative bacteria, comprising proteoglycans, which can be obtained using a method for preparing a membrane fraction as described in claim 4 or 5.

25/ The pharmaceutical composition as claimed in claim 24, characterized in that said Gram-negative bacterium is Klebsiella *pneumoniae*.

26/ The pharmaceutical composition as claimed in claim 24 or 25, characterized in that it is combined with an anticancer treatment by chemotherapy and/or by radiotherapy.

27/ The pharmaceutical composition as claimed in claim 26, characterized in that it contains an anticancer compound as a combination product for use which is simultaneous, separate or spread out over time.

28/ The pharmaceutical composition as claimed in claim 27, characterized in that said anticancer compound

is chosen from protease inhibitors or from compounds with anti-angiogenic activity.

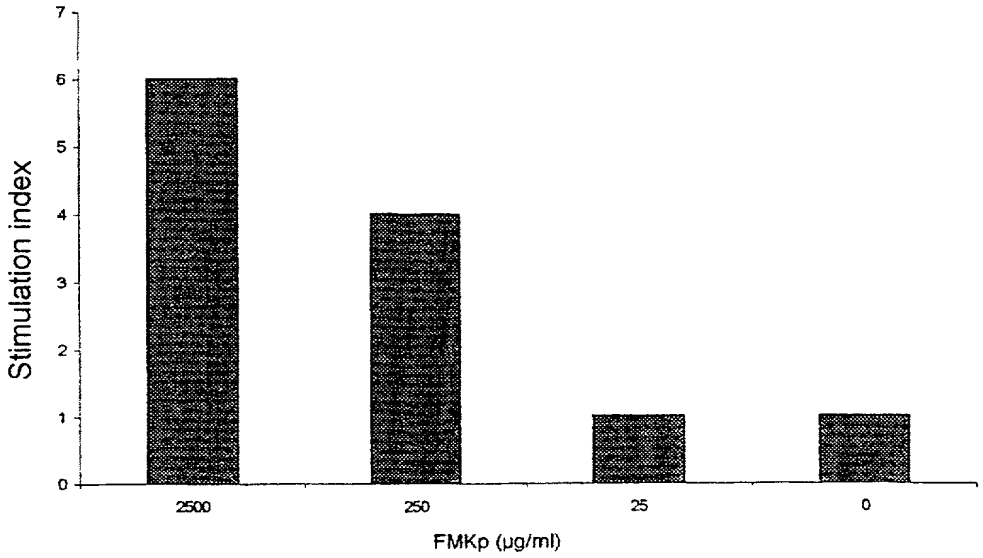
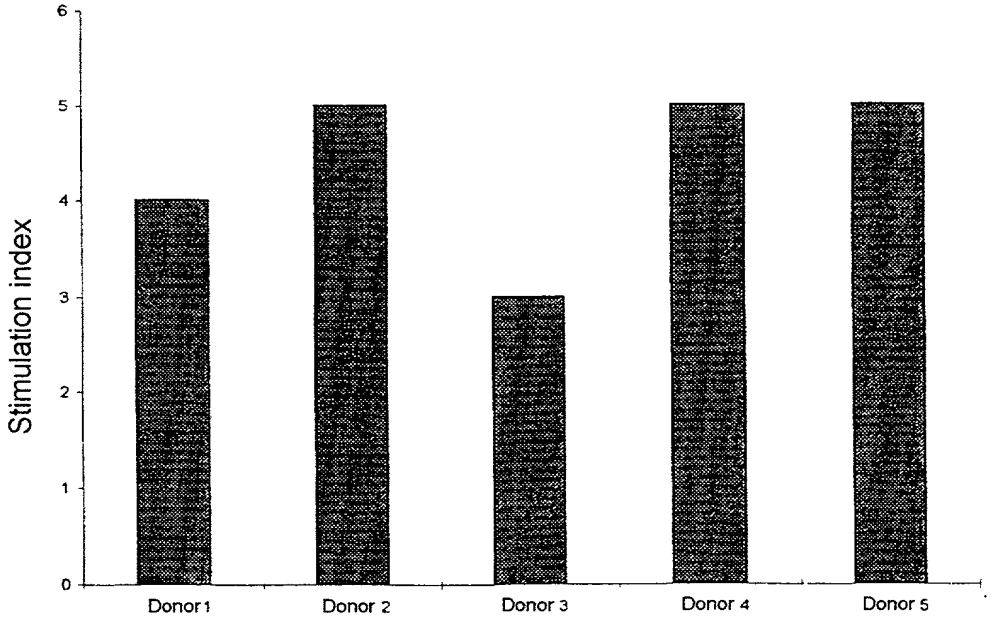


FIGURE 1



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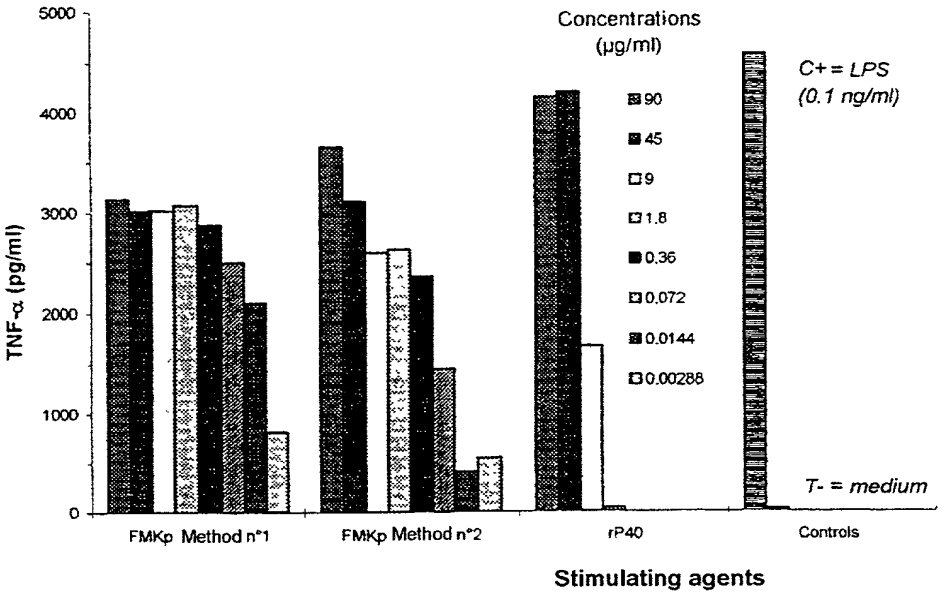

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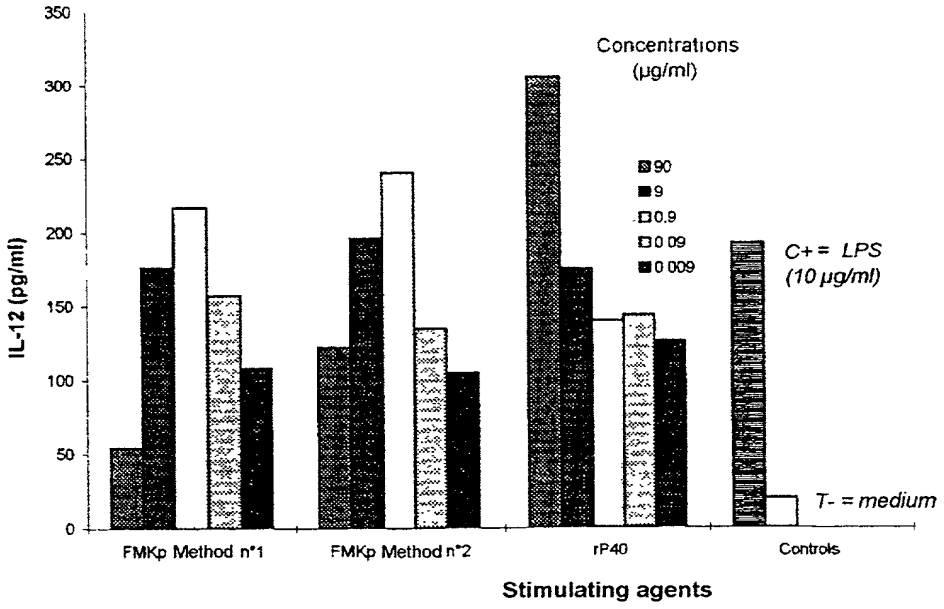

FIGURE 4

Docket Number: PF95PCTSEQ/dln

DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below, next to my name, and

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled **IMMUNOSTIMULANT BACTERIAL MEMBRANE FRACTIONS IN CANCER TREATMENT**

the specification of which (check one of the following)

× is attached hereto

was filed on _____ as
Application Serial No. _____
And was amended on _____
(if applicable)

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor(s) certificate having a filing date before that of the application on which priority is claimed:

Application Serial Number	Country	Filing Date (Day/Month/Year)	Priority Claimed (yes/no)
99 03154	FRANCE	15/March/1999	Yes

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application.

Docket Number: _____

(Application Serial No.)	(Filing Date)	(Status xxxxxxxd, pendingxxxxxxxxx
PCT/FR00/00623	15.03.2000	
(Application Serial No.)	(Filing Date)	(Status - patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status - patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

I hereby appoint the following person registered to practice before the Patent and Trademark Office as my attorney with full power of substitution and revocation to prosecute this application and all divisions and continuations thereof and to transact all business in the Patent and Trademark Office connected therewith and request that all correspondence be sent to him at the mailing address hereafter given:

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	Kalamazoo, MI 49007	

25666
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Name

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616/382-0030

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Docket Number: _____

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Inventor's Signature: Date:

Residence: Citizenship:
(City, State) (Country)

Post Office Address:

Full Name of Sixth/Joint Inventor:

Inventor's Signature: Date:

Residence: Citizenship:
(City, State) (Country)

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Gly Met Val Trp Arg Ala Asp Ser Lys Gly Asn Tyr Ala Ser Thr Gly
        115                 120                 125

Val Ser Arg Ser Glu His Asp Thr Gly Val Ser Pro Val Phe Ala Gly
    130                 135                 140

Gly Val Glu Trp Ala Val Thr Arg Asp Ile Ala Thr Arg Leu Glu Tyr
145                 150                 155                 160

Gln Trp Val Asn Asn Ile Gly Asp Ala Gly Thr Val Gly Thr Arg Pro
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Asp Asn Gly Met Leu Ser Leu Gly Val Ser Tyr Arg Phe Gly Gln Glu
            180                 185                 190

Asp Ala Ala Pro Val Val Ala Pro Ala Pro Ala Pro Ala Pro Glu Val
        195                 200                 205

Ala Thr Lys His Phe Thr Leu Lys Ser Asp Val Leu Phe Asn Phe Asn
    210                 215                 220

Lys Ala Thr Leu Lys Pro Glu Gly Gln Gln Ala Leu Asp Gln Leu Tyr
225                 230                 235                 240

Thr Gln Leu Ser Asn Met Asp Pro Lys Asp Gly Ser Ala Val Val Leu
                245                 250                 255
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Gly Tyr Thr Asp Arg Ile Gly Ser Glu Ala Tyr Asn Gln Gln Leu Ser
260 265 270

Glu Lys Arg Ala Gln Ser Val Val Asp Tyr Leu Val Ala Lys Gly Ile
275 280 285

Pro Ala Gly Lys Ile Ser Ala Arg Gly Met Gly Glu Ser Asn Pro Val
290 295 300

Thr Gly Asn Thr Cys Asp Asn Val Lys Ala Arg Ala Ala Leu Ile Asp
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Pro Thr Thr Lys Gln Arg Gln Asn Lys Pro Pro Asn Lys Pro Asn Asn
20 25 30

gat ttc cat ttc gaa gtg ttc aac ttc gtg ccg tgc agc atc tgc agc 144
Asp Phe His Phe Glu Val Phe Asn Phe Val Pro Cys Ser Ile Cys Ser
35 40 45

aac aac ccg acc tgc tgg gcg atc tgc aaa cgt atc ccg aac aaa aaa 192
Asn Asn Pro Thr Cys Trp Ala Ile Cys Lys Arg Ile Pro Asn Lys Lys
50 55 60

ccg ggc aaa aaa acc acg acc aaa ccg acc aaa aaa ccg acc ttc aaa 240
Pro Gly Lys Lys Thr Thr Thr Lys Pro Thr Lys Lys Pro Thr Phe Lys
65 70 75 80

acc acc aaa aaa gat cat aaa ccg cag acc acc aaa ccg aaa gaa gtg 288
Thr Thr Lys Lys Asp His Lys Pro Gln Thr Thr Lys Pro Lys Glu Val
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<213> Klebsiella pneumoniae

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            20                  25                  30

Asp Phe His Phe Glu Val Phe Asn Phe Val Pro Cys Ser Ile Cys Ser
        35                  40                  45

Asn Asn Pro Thr Cys Trp Ala Ile Cys Lys Arg Ile Pro Asn Lys Lys
    50                  55                  60

Pro Gly Lys Lys Thr Thr Thr Lys Pro Thr Lys Lys Pro Thr Phe Lys
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Thr Thr Lys Lys Asp His Lys Pro Gln Thr Thr Lys Pro Lys Glu Val
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Pro Thr Thr Lys Pro
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SEQUENCE LISTING

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<120> USE OF BACTERIAL MEMBRANE FRACTIONS WITH IMMUNOSTIMULANT ACTIVITY IN THE TREATMENT OF CANCERS, METHODS FOR PREPARING THEM AND THE PHARMACEUTICAL COMPOSITIONS CONTAINING THEM

<130> D17974

<140> FR 99 03154

<141> 1999-03-15

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tat gca ggt ggt aaa ctg ggt tgg tcc cag tat cac gac acc ggt ttc   96
Tyr Ala Gly Gly Lys Leu Gly Trp Ser Gln Tyr His Asp Thr Gly Phe
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tac ggt aac ggt ttc cag aac aac aac ggt ccg acc cgt aac gat cag   144
Tyr Gly Asn Gly Phe Gln Asn Asn Asn Gly Pro Thr Arg Asn Asp Gln
         35                  40                  45

ctt ggt gct ggt gcg ttc ggt ggt tac cag gtt aac ccg tac ctc ggt   192
Leu Gly Ala Gly Ala Phe Gly Gly Tyr Gln Val Asn Pro Tyr Leu Gly
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Val Asp Asn Gly Ala Phe Lys Ala Gln Gly Val Gln Leu Thr Ala Lys
                85                  90                  95
ctg ggt tac ccg atc act gac gat ctg gac atc tac acc cgt ctg ggc   336
Leu Gly Tyr Pro Ile Thr Asp Asp Leu Asp Ile Tyr Thr Arg Leu Gly
            100                 105                 110
ggc atg gtt tgg cgc gct gac tcc aaa ggc aac tac gct tct acc ggc   384
Gly Met Val Trp Arg Ala Asp Ser Lys Gly Asn Tyr Ala Ser Thr Gly
        115                 120                 125
gtt tcc cgt agc gaa cac gac act ggc gtt tcc cca gta ttt gct ggc   432
Val Ser Arg Ser Glu His Asp Thr Gly Val Ser Pro Val Phe Ala Gly
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ggc gta gag tgg gct gtt act cgt gac atc gct acc cgt ctg gaa tac   480
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145                 150                 155                 160
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Gln Trp Val Asn Asn Ile Gly Asp Ala Gly Thr Val Gly Thr Arg Pro
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gat aac ggc atg ctg agc ctg ggc gtt tcc tac cgc ttc ggt cag gaa   576
Asp Asn Gly Met Leu Ser Leu Gly Val Ser Tyr Arg Phe Gly Gln Glu
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Lys Ala Thr Leu Lys Pro Glu Gly Gln Gln Ala Leu Asp Gln Leu Tyr
225                 230                 235                 240
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ggc tac acc gac cgc atc ggt tcc gaa gct tac aac cag cag ctg tct   816
Gly Tyr Thr Asp Arg Ile Gly Ser Glu Ala Tyr Asn Gln Gln Leu Ser
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gag aaa cgt gct cag tcc gtt gtt gac tac ctg gtt gct aaa ggc atc   864
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Pro Ala Gly Lys Ile Ser Ala Arg Gly Met Gly Glu Ser Asn Pro Val
    290                 295                 300
act ggc aac acc tgt gac aac gtg aaa gct cgc gct gcc ctg atc gat   960
Thr Gly Asn Thr Cys Asp Asn Val Lys Ala Arg Ala Ala Leu Ile Asp
305                 310                 315                 320
tgc ctg gct ccg gat cgt cgt gta gag atc gaa gtt aaa ggc tac aaa   1008
Cys Leu Ala Pro Asp Arg Arg Val Glu Ile Glu Val Lys Gly Tyr Lys
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         35                  40                  45
Leu Gly Ala Gly Ala Phe Gly Gly Tyr Gln Val Asn Pro Tyr Leu Gly
     50                  55                  60
Phe Glu Met Gly Tyr Asp Trp Leu Gly Arg Met Ala Tyr Lys Gly Ser
 65                  70                  75                  80
Val Asp Asn Gly Ala Phe Lys Ala Gln Gly Val Gln Leu Thr Ala Lys
                 85                  90                  95
Leu Gly Tyr Pro Ile Thr Asp Asp Leu Asp Ile Tyr Thr Arg Leu Gly
            100                 105                 110
Gly Met Val Trp Arg Ala Asp Ser Lys Gly Asn Tyr Ala Ser Thr Gly
        115                 120                 125
Val Ser Arg Ser Glu His Asp Thr Gly Val Ser Pro Val Phe Ala Gly
    130                 135                 140
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Gly Val Glu Trp Ala Val Thr Arg Asp Ile Ala Thr Arg Leu Glu Tyr
145   150   155   160

Gln Trp Val Asn Asn Ile Gly Asp Ala Gly Thr Val Gly Thr Arg Pro
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Asp Asn Gly Met Leu Ser Leu Gly Val Ser Tyr Arg Phe Gly Gln Glu
            180   185   190

Asp Ala Ala Pro Val Val Ala Pro Ala Pro Ala Pro Ala Pro Glu Val
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Ala Thr Lys His Phe Thr Leu Lys Ser Asp Val Leu Phe Asn Phe Asn
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Lys Ala Thr Leu Lys Pro Glu Gly Gln Gln Ala Leu Asp Gln Leu Tyr
225   230   235   240

Thr Gln Leu Ser Asn Met Asp Pro Lys Asp Gly Ser Ala Val Val Leu
                245   250   255

Gly Tyr Thr Asp Arg Ile Gly Ser Glu Ala Tyr Asn Gln Gln Leu Ser
            260   265   270

Glu Lys Arg Ala Gln Ser Val Val Asp Tyr Leu Val Ala Lys Gly Ile
        275   280   285

Pro Ala Gly Lys Ile Ser Ala Arg Gly Met Gly Glu Ser Asn Pro Val
    290   295   300

Thr Gly Asn Thr Cys Asp Asn Val Lys Ala Arg Ala Ala Leu Ile Asp
305   310   315   320

Cys Leu Ala Pro Asp Arg Arg Val Glu Ile Glu Val Lys Gly Tyr Lys
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Glu Val Val Thr Gln Pro Ala Gly
            340